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# QUANTITATION OF SPECIFIC PROTEINS IN BLISTER FLUID*

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## ABSTRACT

Radial immunodiffusion is used to determine specific proteins in friction blister fluid and cantharidin blister fluid of human volunteers. Four proteins (albumin, fibrinogen, immunoglobin IgG and IgM) were determined in blister fluid pooled 2, 4 and 21 hours after blistering. The same proteins were also determined in cantharidin blister fluid from the back and palm. The concentration of a specific protein in friction blister fluid relative to the concentration in plasma is approximately the same for albumin, IgG and IgM while essentially no fibrinogen is found in this blister fluid. Relative concentrations of albumin, IgG and IgM are much larger in cantharidin blister fluid while a small amount of fibrinogen can be determined. This suggests that damage from cantharidin to epidermis and dermis is much more pronounced in the cantharidin blister than in the friction blister. It is also shown that fibrinogen is not destroyed by the friction blister fluid. Implications of the findings are discussed.

Accumulation of fluid in a bulla of the skin requires two distinct processes: 1) separation of epidermal and/or dermal cells from each other so that an extracellular space is created and 2) the amount of fluid coming from blood vessels per unit of time must be larger than the resorption by the lymph vessels and the venules. In the process of separation cells are damaged, intracellular soluble proteins released and enzymes liberated. Soluble proteins filtered from the blood are thus mixed with cellular proteins.

The composition of serum and blister fluid has been determined by electrophoresis. Comparisons between serum and blister fluids have been made for cantharidin blisters (1), friction blisters (2), pemphigus vulgaris (3, 4). However, interpretation of electrophoretic patterns of blister fluids is difficult since even subfractions of human plasma are very complicated mixtures; they appear homogeneous simply because their components show the same migration velocity under the chosen conditions. It should also be pointed out that the calculation of relative concentrations of proteins from electrophoretic experiments gives incomplete or even misleading information, since any change in the absolute concentration of one protein fraction produces a change in the relative concentration of all components. Consequently the comparison of blister fluid with serum by electrophoretic methods is of limited usefulness.

It has been shown that fluid in the subepidermal blisters of erythema multiforme and bullous pemphigoid always clots (5), indicating that fibrinogen and other components of the blood clotting system have passed through the capillary wall. This suggests that the blood vessels become abnormally permeable in erythema multiforme and bullous pemphigoid, and that the membrane between dermis and epidermis also leaks proteins. This explains why fibrinogen and other large protein molecules can filter into the blister space.

In contrast to blister fluid in pemphigus vulgaris and the fluid in thermal blisters (5), the fluid of friction blisters does not clot. This suggests that little irreversible damage is produced in the blood vessels and/or that the basement membrane may not be injured in these blisters. Consequently large protein molecules may not appear in these blister fluids. However, it is also possible that one of several components, necessary for clot formation, are destroyed by activated enzymes.

In this study a new approach is taken to determine proteins in the fluid of friction blisters and of cantharidin blisters. Proteins are quantitated by single radial immunodiffusion (6, 7, 8). This micro method is specific and quantitative for a particular protein. In this paper concentrations of albumin, fibrinogen

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and immunoglobulins IgG and IgM are reported.

## METHODS AND MATERIALS

*Preparation of blister.* Friction blisters were produced on the palms of volunteers. Blister fluid was collected and pooled. Collection was between 0 and 2 hours, 0 and 4 hours and 0 and 21 hours respectively.

Cantharidin blisters were produced on the back and on the palm of volunteers.

The following reagents were commercially available: Fibrinogen (human), Cutter Laboratories, Berkeley, California; Albumin, normal human serum, Courtland Laboratories; Antiserum to Human Albumin, to Fibrinogen to Immunoglobulin IgG and IgM and Behringwerke standardized and stabilized human serum were purchased from Certified Blood Donor Service, Inc., Woodbury, New York. In general, modifications (6, 7) of the procedure of Mancini *et al.* (8) were used.

*Preparation of the agar plates.* A borate buffer solution, pH 8.4, was made by dissolving 37.2 g of boric acid, 4.0 g of sodium hydroxide and 18 g of sodium chloride in 2 liters of water. To 100 ml of this borate buffer were added 2.5 g of Special Agar Noble (Difco 0142-02), the suspension was placed in a boiling water bath and stirred until all the agar was dissolved. Distilled water was added to replace losses due to evaporation and 8 ml of this stock solution was poured into test tubes, the tubes stoppered and stored at 4°C.

Eight ml of solidified agar was melted and 1.95 to 1.4 ml borate buffer added. The solution was allowed to cool to 58°C. Antiserum was warmed to 57°C and 0.05 to 0.6 ml added to the agar solution. The solution was mixed by aspirating into a prewarmed pipette making sure that no bubbles were produced.

The agar plates were hydrated over a Ringer solution for about 1 hour. Circular wells were punched with a cutter of about 2.0 mm diameter. The small cylinder of gel, punched out by the cutter, was removed by suction. Each of the small cylindrical holes was filled with 4 microliters of antigen solution. The plates were stored for 1 hour over Ringer solution until the solution had diffused into the agar.

*Incubation, washing, and staining of agar plates.* The agar plates were placed on the bottom part of a petri dish and mineral oil added to cover the plate. A crystal of thymol was added to the mineral oil to suppress bacterial and fungal growth. The petri dish was covered with the top dish and the petri dish assembly incubated in a horizontal position at 37°C for up to 7 days.

The agar plates were removed from the petri dishes and rinsed two times with petroleum ether. Thereafer the plates were submerged in normal saline for 3 hours. Washing was repeated two times. Finally the plates were immersed in distilled water overnight.

A dye solution was made by dissolving 2.2 g Naphthol Blue Black in 100 ml methanol and adding 100 and distilled water and 20 ml glacial acetic acid. The agar plates were immersed for 2 minutes in the dye-solution and then rinsed with methanol-water-acetic acid solution (10:10:2) until clear.

*Quantitation of color spots.* The spot image of the agar plate was projected onto heavy white paper with a 3¼ inch by 4 inch lantern slide projector from a distance of 100 centimeters. The colored spots were carefully marked, cut out and weighed on an analytical balance. From the spot weight, the weight of the area of the circular well was subtracted.

*Evaluation of data.* Each blister fluid sample was incubated in duplicate in at least 3 different concentrations and 3 standard samples of an antigen included on a plate. The regression line, relating dilution and spot weight, the regression coefficient and correlation coefficient were calculated. Results were only considered if an apparent positive correlation, which was real and strong, was calculated.

## RESULTS

The concentration of albumin, IgG, IgM and fibrinogen are listed in Table I for blood plasma (9, 10, 11, 12). The standard deviation for each value is also given. Values of IgG and IgM are from several investigators. A "best" value has not been determined.

Since friction blisters were pooled for the 2 hour, 4 hour and 21 hour time interval, protein values represent mean values of a small number of samples. The standard deviation may, therfore, be rather large. A more detailed study relating friction blister content to plasma content of individual volunteers, taking into account circadian fluctuation in proteins, is in progress. For purpose of comparison, concentrations of a specific protein relative to the concentration in plasma are tabulated in Table II.

It should be noted that the ratios of the concentrations of albumin, IgG and IgM in friction blister fluid are of the same order of magnitude as in plasma, whereas fibrinogen is absent. The relative protein concentrations for cantharidin blisters are much higher. It is possible that a significant difference in relative protein concentration exists for the proteins of the fluid from the back and the palm. However, the protein concentration in the cantharidin blister is much greater than in the friction blister fluid.

*Fibrinogenolytic activity of friction blister*

TABLE I

*Concentration of protein in blister fluid (mg/ml)*

| | Plasma mean | $\sigma$ | Reference | Friction blister | | | Cantharidin | |
|---|---|---|---|---|---|---|---|---|
| | | | | 2 hrs.[a] | 4 hrs.[b] | 21 hrs.[c] | Back[c] | Palm[c] |
| Albumin | 40.6 | 3.4 | 9 | 4.6 | 3.6 | 6.8 | 12.6 | 28.7 |
| IgG | 10.9 | 1.7 | 9 | 1.5 | 0.6 | 1.3 | 3.6 | 7.0 |
| | 12.4 | 2.2 | 10 | | | | | |
| | 1.23 | 0.35 | 10 | | | | | |
| IgM | 0.74 | 0.31 | 11 | 0.08 | 0.17 | 0.06 | 0.6 | 0.7 |
| | 0.67 | 0.28 | 9 | | | | | |
| Fibrinogen | 3.4 | 0.6 | 12 | 0.00 | 0.00 | 0.01 | 0.12 | 0.13 |

[a] Samples from 2 volunteers.
[b] Samples from 5 volunteers.
[c] Samples from 5 volunteers.

*fluid*. One hundred microliters of friction blister fluid and 5 $\mu$l of a solution of 20 mg/ml fibrinogen were incubated in duplicate at 37°C. At 2, 6 and 24 hours, 4 microliters of this mixture were added to a radial immunodiffusion plate. The plates were then incubated for 7 days and processed as described under Methods. The spot weight of the areas are given in Table III. The spot weights indicate that under these conditions fibrinogen is not destroyed by the blister fluid.

TABLE II

*Relative concentration of protein in blister fluid*

Plasma = 1.0

| | Friction blister | | | Cantharidin | |
|---|---|---|---|---|---|
| | 2 hrs. | 4 hrs. | 21 hrs. | Back | Palm |
| Albumin | 0.11 | 0.09 | 0.17 | 0.31 | 0.70 |
| IgG | 0.11 | 0.05 | 0.10 | 0.28 | 0.55 |
| IgM | 0.11 | 0.24 | 0.08 | 0.85 | 1.0 |
| Fibrinogen | 0.00 | 0.00 | 0.00 | 0.03 | 0.04 |

TABLE III

*Weight of paper cut out after incubation of fibrinogen with friction blister fluid*

| Time of incubation | Paper weight (mg) per 3.81 $\mu$g fibrinogen |
|---|---|
| 2 hr. | 262.5 |
| | 270.5 |
| 6 hr. | 254.6 |
| | 255.0 |
| 24 hr. | 268.5 |
| | 269.2 |

## DISCUSSION

Morphologically, friction blisters and cantharidin blisters are classified as intraepidermal blisters. Without exception, the blister cleft at the moment of blistering always appears within the malpighian layer and the cleavage occurs at its upper level. The dermis of blisters without secondary infection shows little inflammatory infiltrate (13). This would suggest that the protein composition could be identical in the two blister fluids. However, it has been shown with the electron microscope (14) that cantharidin damages a variety of membranes resulting in severe cellular injury to the epidermis. Because of solubility and penetration of this drug, damage may not be restricted to the epidermis but subepidermal structures may also be affected. In contrast, it appears that subepidermal structures are not damaged in friction blisters (13). Thus, it may be expected that the protein composition in cantharidin blister fluid is different from that of the friction blister. This difference is true in relation to fibrinogen, which cannot be detected in the friction blister fluid but can readily be determined in cantharidin blister fluid.

Quantitatively, the concentration of protein might also be larger for the cantharidin blister fluid than for the friction blister fluid. Our measurements, reported in Table I, show a significantly higher concentraton of albumin, IgG and IgM in cantharidin blister fluid than in friction blister fluid. Passage of serum proteins into the cantharidin blister space is thus much more pronounced than passage into the friction blister space.

The concentration of fibrinogen (MW 340,000) in friction blister fluid 2, 4 and possibly 21 hours after blistering is less than the minimal detectable concentration of 0.01 mg/ml. In constrast, IgM, a much larger protein (MW 800,000 – 1,000,000), is readily detectable in friction blister fluid. Consequently, size of the molecule is not the limiting factor for exclusion of fibrinogen from the blister fluid. This suggests that fibrinogen is either destroyed or is excluded by some specific process. However, no destruction of fibrinogen could be detected when this protein was incubated with blister fluid for up to 24 hours. This suggests that a specific process excludes fibrinogen from the blister space.

Determination of fibrinogen (MW 340,000) in lymph fluid of the thoracic duct (19) indicates that this protein can pass from some blood vessels into the corium and the lymph vessel. Prothrombin, a much smaller molecule (MW 68,500), should also readily pass into the corium. If conversion of inactive prothrombin into thrombin were possible in the corium, then the liberated thrombin would transform the fibrinogen into fibrin.

Depots of fibrin have been described in several electron-microscopic investigations. For instance, Wilgram showed precipitates of proteins between collagen fibrils and collagen bundles in bullous pemphigoid (14). When properly oriented, portions of these precipitates showed a periodicity of 220 Å indicative of fibrin. Majno and Palade showed, within one minute after local injection of histamine or serotonin, gaps in the endothelial lining of certain blood vessels (15). They also found filaments exhibiting the typical periodicity of fibrin (220 Å). The absence of fibrinogen from friction blister fluid is thus consistent with the suggestion that fibrinogen is transformed into fibrin in the corium. Such a deposition of fibrin may or may not occur in the case of cantharidin blisters since fibrinogen can readily be determined in low concentrations in cantharidin blister fluid.

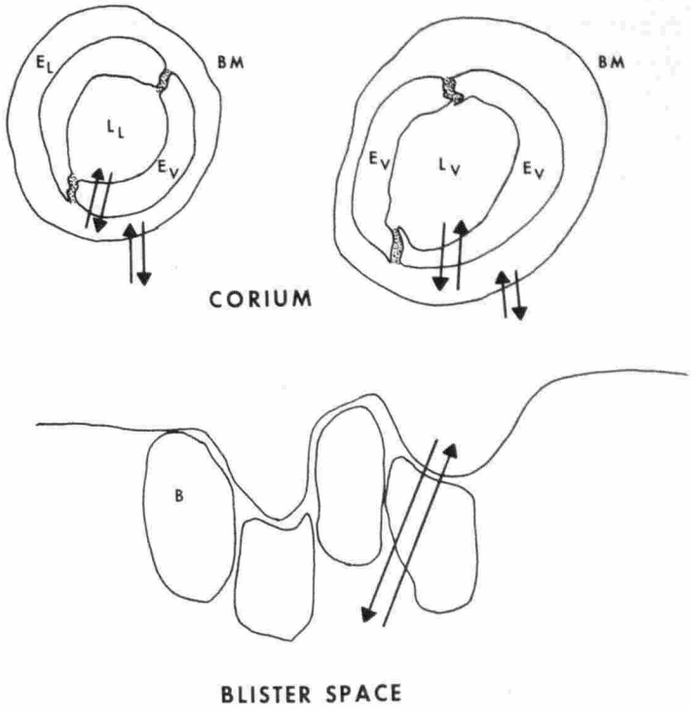


FIGURE. Schematics of protein transfer in the skin $L_V$ and $L_L$ are the lumen of blood vessel and lymph vessel response. $E_V$ and $E_I$ are the endothelial cells of blood vessel and lymph vessel response, B.M. basement membrane, B basal cell of the epidermis.

Albumin and fibrinogen have been determined in the thoracic lymph fluid (19). However, this need not necessarily mean that the small lymphatic vessels of the skin contain either one of these proteins in a significant concentration.

Mild cellular damage and leaking capillaries appear when skin is heated to 54°C for 20 seconds (16). This particular form of vascular response is a common pattern of increased permeability after many kinds of injury. Intercellular gaps are present in the endothelium but the endothelial cells are unchanged. A large amount of amorphous material (presumably protein) is often piled against the basement membrane at the epidermo-dermal boundary. Other studies indicate that the basement membrane is permeable to carbon particles of 300 Å diameter (17). Perhaps a similar response is evoked by frictional forces. Thus, plasma proteins may permeate into the corium or, after passing the membrane separating corium and epidermis, into the blister space.

The composition and concentration of proteins in the blister space are thus intimately

related to rate constants for transfer of material between compartments as indicated schematically in the Figure. Mathematically speaking, this is nothing more than a mammillary system where one central compartment (the corium) is surrounded by a series of peripheral ones (lymph vessel $L_l$, blood vessel $L_v$ and blister space) (18).

If the rate determining process for transfer of protein from the lumen of the blood vessel $L_v$ to the blister space were governed by the laws of diffusion it would be expected that diffusion were some inverse function of the size of the molecule (a rigorous mathematical solution of this complex problem has not been worked out). Consequently it would follow that the small molecule albumin should be present in the blister fluid at short periods after blistering in a much higher concentration than a molecule of the size of IgM. The relative protein concentrations for albumin, IgG and IgM indicate, however, that molecular size is unimportant (Table II). Consequently the rate determining step for transfer of proteins from the blood to the blister space may not be dependent on diffusion. Rather, the rate limiting transfer may occur in packages, i.e., a vacuole of fluid may be transferred towards the blister space (pinocytosis, passage along tight junctions of the endothelial cells etc.).

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